Biodegradable Photothermal and pH Responsive Calcium Carbonate@Phospholipid@Acetalated Dextran Hybrid Platform for Advancing Biomedical Applications

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A biodegradable multifunctional carrier for combination therapy with high efficiency and low side effect is essential for effective cancer treatment and for advancing biomedical applications. Therapeutics combination could reduce multidrug resistance and minimize doses through synergism. This study develops biodegradable gold nanorods@calcium carbonate particles coated with pH-responsive acetalated dextran and phospholipid as an advanced platform for the incorporation of versatile molecular targeted therapeutics, including hydrophilic and hydrophobic drugs, as well as the model enzyme, green fluorescent protein, or antibody. The developed calcium carbonate based hybrid particles show good biocompatibility, stability with photothermal, and pH responsiveness, which protect the payloads from premature release, and maintain the enzyme activity. The therapeutics co-loaded CaCO\(_3\) based hybrid particles efficiently induce cancer cell death and reduce the multidrug resistance and HER2 expression with synergism. The photothermal effects promote ultrafast therapeutics release and induce significant cytotoxicity. Importantly, Anti-HER2 antibody or HER2 targeted therapeutic is more effective in reducing HER2 expression when combined with drug or drugs via synergism. Overall, the cheap and simply manufactured biodegradable hybrid platform has great potential for advancing biomedical applications, including targeted photothermal combination therapy by co-delivery of different types of therapeutics, including molecular targeted drugs, antibodies, and enzymes.

1. Introduction

Combination therapy is currently the standard route for effective cancer treatment to suppress and reduce multidrug resistance caused by single drug, and can minimize dose by synergism. The successful development of biocompatible co-delivery platform for therapeutics combination is still to be achieved.[1] In many cases, different chemical drugs and other types of therapeutics, such as enzymes, antibodies, and nucleic acids, are given in the same formulation to achieve synergistic effect for combination therapy.[2,3] For cancer treatment,[4,5] the traditional cytotoxic drugs and molecular targeted therapies[6-9] are still most effective when combined together.[6,7] Synergistic drugs combination is beneficial in the view of retarding the occurrence of resistant cell lines and wide coverage against multiple cell lines, resulting in a maximum cancer cell death with an acceptable toxicity.[8,9] The current drug delivery tools for multiple drug combinations are far from perfect,[10] which are greatly limited.
with the co-delivery efficiency and the biocompatibility of the carrier themselves. One effective way to enhance drug efficacy and reduce its side effect is to simultaneously encapsulate them into carriers.\cite{11,12} Many different types of nanoparticles and microparticles have been extensively studied as drug delivery systems.\cite{13–19} For example, polymersomes and liposomes have been fabricated to simultaneously encapsulate hydrophilic and hydrophobic therapeutics in the aqueous core and the oil shell.\cite{20} In addition, lipid particles, polymer based particles, magnetic responsive particles, porous materials, and inorganic particles are popular tools for drug delivery.\cite{21–24}

An ideal advanced drug formulation should have proper properties such as high stability and good biocompatibility and can produce a favorable pharmacokinetic drug profile for the encapsulated drugs. In addition, an optimum drug delivery platform should be cheap, which is realistic for commercialization and scale-up. Thus, it is very important to develop a simple one-step solvent-free and cost-effective platform to produce generic biocompatible multifunctional carrier for co-delivery of therapeutics to fully exploit synergistic therapeutics combination. Calcium carbonate was reported to be useful as an intranasal carrier of insulin, granulocyte-colony stimulating factor, and betamethasone phosphate due to its promising properties, such as cheap manufacture, easy production, simple delivery, and slow biodegradability.\cite{25} The sustained release of hydrophilic drugs and bioactive proteins was confirmed both in vitro and in vivo in this system.\cite{26} Acetalated dextran (AcDX) is a pH responsive and biocompatible coating material, which degrades at acidic pH values that occur at the site of cancer or inside the cells in lysosomes.\cite{27,28} The sustained release of hydrophilic drugs and bioactive proteins was confirmed both in vitro and in vivo in this system.\cite{26} Acetalated dextran (AcDX) is a pH responsive and biocompatible coating material, which degrades at acidic pH values that occur at the site of cancer or inside the cells in lysosomes.\cite{27,28}

Doxorubicin (DOX) is one of the most popular chemotherapy drugs for many different types of cancer treatments; however, it suffered with low oral bioavailability.\cite{29} DOX and hydrophobic drugs 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) are reported to have synergism effect on breast cancer cells and small cell lung cancer.\cite{30} Afatinib kills HER2 positive breast cancer cells and spare normal cells.\cite{31} Amylase is a very widely used enzyme that exists in human, and in this study was chosen as a reporter on the enzyme stability inside the calcium carbonate. Anti-HER2 antibody can inhibit the HER2 protein expression.

In this study, the biodegradable photothermal responsive calcium carbonate particles coated with pH responsive acetalated dextran and phospholipid are synthesized as carrier for co-incorporation of versatile molecular targeted therapeutics including hydrophilic drug DOX, hydrophobic drugs 17-AAG and afatinib, and a model enzyme amylase or green fluorescent protein FITC-BSA or anti-HER2 antibody.

2. Results and Discussion

2.1. Synthesize and Characterization of the Hybrid Particles

We synthesized CaCO₃ particles, and amylose, FITC-BSA, antibody, DOX alone, 17-AAG and afatinib alone, and DOX+17–AAG and DOX+afatinib combinations, and gold nanorods (AuNRs) were incorporated into CaCO₃ particles with and without coating of phospholipid and AcDX by the simple mechanical stirring method.\cite{32} Scheme 1 shows the biocompatible CaCO₃@POPC-AcDX hybrid platform with photothermal and pH responsiveness for advancing biomedical applications by co-delivery of versatile therapeutics, enzyme and antibody. The diameter of the CaCO₃ particles was around 100 nm at the mixing rate of 1600 rpm and 400 nm at the mixing rate of 600 rpm. The particle size distribution was narrow and the drug loading efficiency was up to 86% at the optimum ratio (1:1.3) between Ca²⁺ and CO₃²⁻. The scanning electron microscopy (SEM) images show that the morphology of CaCO₃ particles is different when different payloads are loaded (Figure 1). Pure CaCO₃ particles are round balls with CaCO₃ molecules on the surface (Figure 1a); when DOX is loaded it seems that the drug partially accumulates at the surface (Figure 1b). The Fourier transform infrared spectroscopy (FTIR) results further confirmed the presence of DOX on surface (Figure S1a, Supporting Information). Peaks corresponding to DOX were observed in the DOX@CaCO₃ sample. When AuNRs are incorporated, some AuNRs point out through the space of CaCO₃ surface (Figure 1d). The different morphology of CaCO₃ was due to the rearrangement of the particles when payloads are incorporated and the partial accumulation of payloads on surface (Figure 1). AcDX and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) coating clearly makes the particles surface smoother and this will be beneficial for the close encapsulation of payloads inside the CaCO₃@AcDX-POPC composite (Figure 1e,f). The phospholipid POPC coating would enhance the biocompatibility of the hybrid particles and reduce the side effect of DOX.\cite{33} The SEM image of CaCO₃ incorporating all therapeutics then coated with AcDX-POPC produced at 600 rpm under magnetic stirring is shown.
in Figure 1f. The transmission electron microscopy (TEM) images of CaCO$_3$, AuNR@CaCO$_3$, and AuNR@CaCO$_3$@AcDX-POPC further proved the incorporation of AuNRs and the existence of the biocompatible coating (Figure 1g–i). The produced CaCO$_3$ and the CaCO$_3$ hybrid platform with all payload incorporated then coated with AcDX-POPC showed good monodispersity (Figure 1j,k). The amylase enzyme and FITC-BSA loading efficiency was about 82%, and the loading efficiency of short gold nanorods was about 80%. The maximum loading efficiency of hydrophobic drug was about 70%, which was much lower than that of hydrophilic therapeutics. The FTIR result of CaCO$_3$ incorporating FITC-BSA (Figure S1b, Supporting Information) confirmed the successful incorporation of FITC-BSA into CaCO$_3$ particles.

It is well known that CaCO$_3$ will incorporate therapeutics into its particle structure,$^{[32]}$ thus it is important to prove whether there has a solid state change of the drug after incorporating into particles. We conducted differential scanning calorimetry (DSC) measurements to confirm the solid state of DOX in the CaCO$_3$ particles and the incorporation of DOX into CaCO$_3$ particles. Figure S2 (Supporting Information) shows that the thermal transition of DOX is at 220 °C if it is physically mixed with calcium carbonate particles, but after the incorporation of DOX into CaCO$_3$ particles, the thermal transition is shifted to
The in vitro digestion study conducted on these multi-drug combined hybrid particles at different ratios between the hydrophilic and hydrophobic drugs (Table S2, Supporting Information) showed that about 70%–80% of anticancer drugs remained solubilized in the aqueous phase following the 30 min lipolysis process. There was no significant difference among the formulations, which indicates that CaCO₃ particles did not affect much the digestion process, making them suitable for the simultaneous encapsulation, co-delivery, and release of drugs combination.

The in vitro dynamic dialysis release profile of DOX alone and drug co-loading with 17-AAG and Afatinib in CaCO₃ particles suspensions are shown in Figure 3a. The in vitro release studies have confirmed that no initial burst release was observed and about 60%–70% of drugs were released within 24 h from the CaCO₃ particles suspension in the release medium tested (phosphate buffered saline (PBS), pH 7.4). The release of DOX from CaCO₃ particles suspension was much slower than the dissolution profiles of free DOX in buffer solution. The release rate was controlled by the CaCO₃ particles since both hydrophilic drug DOX and hydrophobic drug 17-AAG and Afatinib showed similar release profiles (Figure 3a). Figure 3b shows the release of DOX from the CaCO₃ particles with and without pH sensitive AcDX and POPC coating at different release medium. After coating, the release of DOX from the CaCO₃@AcDX particles in acidic medium was much faster than that from neutral medium because the AcDX delayed the drug release at pH 7.4 (Figure 3b). The thermal responsive drug release was also investigated. The release profiles of the drug were not changed by the incorporation of AuNRs onto the particles at 37 °C (Figure 3c), while the AuNRs stimulated the drug release at 45 °C (Figure 3c), which confirmed the heat responsiveness of the platform.

Table S3 (Supporting Information) shows the size change when incorporating drug, AuNRs, or coating with AcDX and POPC to CaCO₃. The AcDX layer is sensitive to acidic pH 5.0, and Table S4 (Supporting Information) shows the structural changes of CaCO₃ after the degradation of outer layer at pH 5.0.

2.4. Incorporation of the Amylase and Evaluation of the Enzymatic Activity

The functionality of the enzyme activity by the biocompatible CaCO₃ particles for the model enzyme α-amylase was...
confirmed by using the amylase assay kit at 37 °C. The calculated activity of amylase incorporated into CaCO₃ particles was 98.0 ± 3.5%. The encapsulation efficiency of amylase by CaCO₃ particles was about 82 ± 3.5%. The release profile of amylase from the particles into 3-(N-morpholino) propanesulfonic acid (MOPS/EDTA) buffer under magnetic stirring condition at 37 °C is shown in Figure 3d. The enzyme incorporated in the CaCO₃ particles showed a sustained release, and the activity was almost kept at 100% after releasing from the CaCO₃ (Figure 3d). Incorporation of enzyme by calcium carbonate particles could protect the activity of the enzyme, which indicates the potential incorporation of antibody and immune responsive drug or gene.

2.5. The Release of FITC-BSA and Antibody from CaCO₃ Hybrid Particles

The study of the encapsulation and in vitro release of the model FITC-BSA and fluorescent antibody (Alexa Fluor 488 secondary antibody) by and from the biocompatible CaCO₃ particles with and without gold nanorods was confirmed by using a microplate reader at 37 °C. The encapsulation efficiency of FITC-BSA or fluorescent antibody by CaCO₃ particles with and without 10 × 10⁻⁹ m AuNRs was about 80.2 ± 3.1% and 85.7 ± 3.3%, respectively. The release profile of FITC-BSA and fluorescent antibody from the particles into PBS buffer under magnetic stirring condition at 37 °C is shown in Figure S6 (Supporting Information). The green fluorescent protein (GFP) or fluorescent antibody incorporated in the CaCO₃ particles showed a sustained release compared to other therapeutics we studied before, and gold nanorods do enhance the loading capacity of the GFP or antibody a little bit more compared to CaCO₃ particles.

2.6. Photothermal Release of Therapeutics from CaCO₃ Hybrid Particles

AuNRs have unique optical properties that are exploited for photothermal therapy[35,36] and triggered drug and protein release.[35–37] But as carrier, the quick leakage of therapeutics...
from AuNRs makes it very difficult to have triggered drug release effect. In this study, AuNRs were incorporated into biodegradable CaCO₃ hybrid particles to build up a platform to enable the photothermal responsive drug release. Compared to the previous polymer therapeutics using plasmonic photothermal therapy,[36] our work focused on the photothermal combination therapy with pH responsiveness and HER2 targeting selectivity which could deliver at tumor site directly. To confirm the endorsement of photothermal responsiveness by AuNRs to the CaCO₃ hybrid particles under laser irradiation, the photothermal release of therapeutics from the platform was conducted by laser excitation at 650 nm wavelength at different time intervals. Figure 4 shows that AuNRs endow the platform with photothermal responsiveness and promote the drug ultrafast release under laser irradiation. Figure S3 (Supporting Information) shows the photothermal release of DOX (488 nm) and AuNRs (975 nm) from hybrids after 650 nm laser irradiation detected by UV–vis spectrum at 298 K. The therapeutics release is almost completed within half an hour. The protection of AuNRs in the CaCO₃ hybrid particles shows the great potential in photothermal combination therapy, bioimaging, and biosensing.

2.7. In Vitro Cell Cytotoxicity of CaCO₃ Hybrid Particles

It is well known that DOX is highly toxic to both endothelial and cardiomyocytes cells, which accounts for severe side effects associated with the systemic administration.[3] Thus it is important to create a biocompatible and biodegradable carrier to encapsulate DOX for delivery. The blank CaCO₃@POPC-AcDX suspensions were incubated with HeLa and MCF-7 cells for 24 h at 37 °C; the cell viability was determined by live/dead assay according to our previous work,[33] as shown in Figure 5a. The CaCO₃@POPC-AcDX hybrid particles with and without incorporation of 1 × 10⁻⁹ M AuNRs almost did not show any toxicity to the cells, for which 90% of HeLa and MCF-7 cells survive in the presence of 400 μg mL⁻¹ of CaCO₃@POPC-AcDX incorporating 5 × 10⁻⁹ M AuNRs. This result demonstrates that the synthesized photothermal and pH responsive AuNR@CaCO₃@POPC-AcDX hybrid particles are highly biocompatible. The coating of phospholipid would reduce the side effect of DOX and enhance the biocompatibility of the hybrids according to our previous work.[33,34]

The synergistic killing effect of the multidrug-loaded photothermal and pH responsive AuNR@CaCO₃@POPC-AcDX hybrid platform at different drug concentrations after 24 h incubation at 37 °C is shown in Figure 5b. Both single drug and multiple drugs incorporated into CaCO₃ hybrid particles can effectively inhibit the cell growth, and the drugs combination is killing more cancer cells than the single drug formulations at the same concentration. This confirms the synergism of the drugs combination in the platform. The multidrug-loaded hybrid particle suspensions induced stronger cell viability than the single drug-loaded hybrid particle suspensions, especially when incubated for a longer period of time (Figure 5b). These results show that when two drugs are simultaneously incorporated into the CaCO₃ hybrid particles, the drugs induced synergistic effects. Figure 5c shows the selectively targeted killing of HER2 positive breast cancer SKBR-3 cells by Afatinib and DOX+Afatinib combination since Afatinib itself has the ability to target HER2. Though DOX and 17-AAG combination has remarkable synergistic effect on killing MCF-7 cells, the combination does not show significantly synergism on HER2 positive cells. The combination index of DOX+17-AAG against MCF-7 cells and DOX+afatinib against HER2 positive SKBR-3 cells was calculated for the type and amount of interaction between two or more drugs with respect to the experimental parameters (IC₅₀), as described elsewhere (Figure 5d).[33,38–40] The results indicate that therapeutics combination had a synergistic effect on cell death. The synergism between DOX and 17-AAG on breast cancer MCF-7 cells death was slightly stronger than that of DOX and afatinib on HER2 positive breast cancer SKBR-3 cells death. The different formulations of drugs combination are effective for the treatment of different types of breast cancer. Afatinib as HER2/EGFR dual molecular targeted drug, when combined with DOX, is more effective on killing HER2 positive cancer cells, which shows its great potential in targeted combination therapy. By incorporation of different therapeutics combination into biodegradable AuNR@CaCO₃@POPC-AcDX hybrid particles, each therapeutics formulation is effective to different type of breast cancer which would enhance its efficacy on cancer treatment.

2.8. ATP Cytotoxicity Detection on HeLa Cells Induced by Drugs Combination

To evaluate the enhancement on HeLa cells death induced by drugs combination, the luminescent adenosine triphosphate (ATP) detection cytotoxicity assay was performed after 4 h treatment at 37 °C. Figure 6 shows that the drugs combination induced much more cytotoxicity on HeLa cells than that
of single drug alone, which confirmed the synergism between drugs combination.

2.9. Multidrug Resistance Inhibition

To investigate the enhanced inhibition against multidrug resistant, the in vitro cell viability of single drug (DOX, 17-AAG, and Afatinib) loaded AuNR@CaCO3@POPC hybrid particles for 24 h with HeLa and MCF-7 cells at different concentrations at 37 °C using an AlamarBlue assay (n = 6). The synergistic killing effect of drug-loaded photo-thermal CaCO3@POPC suspensions at different concentrations incubated for 24 h with MCF-7 cells (DOX alone; 17-AAG alone; DOX+17-AAG particles; afatinib alone; DOX+afatinib particles; C_{DOX}/C_{17-AAG} = 2:1; C_{DOX}/C_{Afatinib} = 2:1; and untreated hybrid particles with 5 × 10^{-9} M gold nanorods were set as control). c) The selectivity targeted killing effect of drug-loaded photothermal CaCO3@POPC suspensions at different concentrations incubated for 24 h with SKBR-3 cells (DOX alone; 17-AAG alone; DOX+17-AAG particles; afatinib alone; DOX+afatinib particles; C_{DOX}/C_{17-AAG} = 2:1; C_{DOX}/C_{Afatinib} = 2:1; and untreated hybrid particles with 5 × 10^{-9} M gold nanorods were set as control). The level of significance was evaluated by the Student’s t-test and the probabilities set at *p < 0.05, **p < 0.01, and ***p < 0.001 using DOX alone as control. d) The synergistic effect between drugs combination. Isobologram: Abscissa and ordinate units are the concentrations of drugs DOX and therapeutics 17-AAG on MCF-7 cells, DOX and afatinib on SKBR-3 cells; the drug 17-AAG or afatinib has an IC_{50} (concentration giving 50% inhibition) of 500 a.u. (arbitrary units), IC_{50} of DOX is 100 a.u.

Figure 5. In vitro cell study. a) Cell viability of CaCO3@AcDX-POPC hybrid particles with and without gold nanorods suspensions incubated for 24 h with HeLa and MCF-7 cells at different concentrations at 37 °C using an AlamarBlue assay (n = 6). b) The synergistic killing effect of drug-loaded photo-thermal CaCO3@POPC suspensions at different concentrations incubated for 24 h with MCF-7 cells (DOX alone; 17-AAG alone; DOX+17-AAG particles; afatinib alone; DOX+afatinib particles; C_{DOX}/C_{17-AAG} = 2:1; C_{DOX}/C_{Afatinib} = 2:1; and untreated hybrid particles with 5 × 10^{-9} M gold nanorods were set as control). c) The selectivity targeted killing effect of drug-loaded photothermal CaCO3@POPC suspensions at different concentrations incubated for 24 h with SKBR-3 cells (DOX alone; 17-AAG alone; DOX+17-AAG particles; afatinib alone; DOX+afatinib particles; C_{DOX}/C_{17-AAG} = 2:1; C_{DOX}/C_{Afatinib} = 2:1; and untreated hybrid particles with 5 × 10^{-9} M gold nanorods were set as control). The level of significance was evaluated by the Student’s t-test and the probabilities set at *p < 0.05, **p < 0.01, and ***p < 0.001 using DOX alone as control. d) The synergistic effect between drugs combination. Isobologram: Abscissa and ordinate units are the concentrations of drugs DOX and therapeutics 17-AAG on MCF-7 cells, DOX and afatinib on SKBR-3 cells; the drug 17-AAG or afatinib has an IC_{50} (concentration giving 50% inhibition) of 500 a.u. (arbitrary units), IC_{50} of DOX is 100 a.u.
Figure 6. ATP cytotoxicity detection on HeLa cells. ATP detection kit cytotoxicity assay on HeLa cells by DOX, Afatinib (A), 17-AAG (G), DOX+AAG, DOX+Aafatinib, and DOX+AAG+Aafatinib after 4 h of treatment at 37 °C. (CaCO₃@AcDX-POPC hybrid particles with 10 × 10⁻⁹ M AuNRs set as control; C₉₀₋₀DOX/C₉₀₋₀Afatinib = 2:1; C₉₀₋₀DOX/C₉₀₋₀17-AAG = 2:1; the total drugs concentration is 210 ng mL⁻¹.)

AuNR@CaCO₃@POPC-AcDX exhibited much stronger cytotoxicity on MCF-7/DOX cells after 30 min laser irradiation due to the ultrafast release of therapeutics. The photothermal effects of AuNRs would promote a remarkable speedy multi-drug resistance inhibition for potential photothermal therapy (Figure 7b).

2.10. Human HER2 (Total) and HER2 (pY1248) Study

Human HER2 (Total) ELISA assay is designed to detect and quantify the full-length level of HER2 protein from lysates of human HER2 positive SKBR3 breast cancer cells treated by the drug and drugs combination loaded AuNR@CaCO₃@POPC-AcDX for 6 h independent of its phosphorylation state.

Figure 8a shows that HER2 targeted therapeutics Afatinib as a protein kinase inhibitor does significantly reduce HER2 expression and irreversibly inhibit HER2 kinases and the combination of Afatinib, DOX, and 17-AAG could reduce more HER2 expression on SKBR3 cells than Afatinib, DOX, and 17-AAG at the same concentration. Figure 8b shows that anti-HER2 antibody could effectively decrease HER2 expression and the combination of anti-HER2 antibody with DOX and 17-AAG could reduce more HER2 expression than each single drug at the same concentration. We also performed the Human HER2 (pY1248) ELISA assay to detect and quantify the phosphorylated HER2 (pY1248) protein from lysates of human HER2 positive SKBR3 breast cancer cells treated by the drug and drugs combination with and without anti-HER2 antibody loaded AuNR@CaCO₃@POPC-AcDX for 16 h. Figure 8c shows that HER2 targeted therapeutics and anti-HER2 antibody could effectively reduce HER2 (pY1248) expression either in combination with each other or with other therapeutics.

2.11. Human Plasma Stability

At the end, we studied the human plasma stability of the CaCO₃ and AuNRs@CaCO₃@AcDX-POPC hybrid. The biocompatible coating clearly improved the stability of the particles in human plasma (Figure S7, Supporting Information) and in addition the layer did not detach from the CaCO₃ particles after 2 h incubation with human plasma (Figure S8, Supporting Information). We also used the plasma stability assay to determine plasma stability of the tested compounds (therapeutics Afatinib) according to standard human plasma stability protocol (Figure S9, Supporting Information). The remaining tested therapeutics incorporated into calcium carbonate coated with polymer AcDX is almost 100%, which is much higher than that of 82% remaining after 120 min incubation without coating polymer. The plasma stability result confirmed the stability of therapeutics incorporated into calcium carbonate particles coating with and without polymer and also proved the advantage of the coating of polymer into calcium carbonate particles.

3. Conclusion

In summary, we develop biodegradable photothermal and pH responsive AuNR@CaCO₃@POPC-AcDX hybrid particles as co-delivery platform for both hydrophilic and hydrophobic molecular targeted therapeutics, as well as for the delivery of enzyme or anti-HER2 antibody with high encapsulation efficiency using a one-step nontoxic solvent-free process. The versatile therapeutics co-loaded AuNR@CaCO₃@POPC-AcDX hybrid platform could effectively inhibit the growth of cancer cells with molecular targeting, enhance cancer cells death by synergism, and reduce the multidrug resistance.
4. Experimental Section

**Materials:** Doxorubicin hydrochloride (DOX), afatinib, Rapamycin, Erlotinib, and 17-AAG were purchased from LC Laboratories with purity >99.0%. POPC was purchased from Avanti Polar Lipid in powder form. Calcium chloride, Na$_2$CO$_3$, Na$_3$PO$_4$, K$_2$CO$_3$, HAuCl$_4$, NaBH$_4$, ascorbic acid, CTAB, FITC-BSA, Alexa Fluor 488 antibody, anti-HER2 antibody, and amylase were purchased from Sigma-Aldrich and used without further purification. Phosphate buffered saline (PBS 1x) without calcium or magnesium was purchased from Lonza. HeLa, M28, SKBR-3, and MCF-7 cell lines were purchased from ATCC. Live/dead assay kit, ATP kit, and Human HER2 ELISA assay kit were purchased from Life technologies. Human HER2 ELISA assay kit were purchased from Life technologies. Highly purified distilled water was obtained from a Millipore Milli-Q system. Molecular biology grade water was purchased from Corning for Human HER2 ELISA assay and ATP assay. All solutions were filtered by a 0.2 µm membrane (Anodisc) prior to the experiments.

**Synthesis of Calcium Carbonate Particles and AcDX, POPC Coating:** The calcium carbonate (CaCO$_3$) particles were synthesized by adding 1 mL of 5 M CaCl$_2$ solution into 5 mL of 1 M K$_2$CO$_3$ and magnetic stirred at 1600 rpm for 10 min. Then, 5 mL of pure water was added into the reactant and formed a suspension. The suspension was centrifuged at 14 000 rpm for 10 min, and the supernatant and precipitate were separated. The obtained precipitate was the formed calcium carbonate particles.

For the AcDX coating, 10 mg mL$^{-1}$ AcDX in ethanol solution was subsequently dropped into calcium carbonate particles reaction solution with magnetic stirring at 600 or 1600 rpm for 10 min. Then the AcDX coated calcium carbonate particles were collected by centrifugation at 14 000 rpm for 10 min and washed with water for three times.

For the phospholipid POPC coating, 100 mg mL$^{-1}$ POPC in ethanol solution was subsequently dropped into calcium carbonate particles reaction solution with magnetic stirring at 600 or 1600 rpm for 10 min. Then POPC coated hybrid CaCO$_3$ particles were collected by centrifugation at 14 000 rpm for 10 min.

**POPC and AcDX coating hybrid particles were formed by adding AcDX and POPC ethanol solution using the above procedure to form the hybrid particles.**

**Synthesis of Gold Nanorods:** AuNRs were synthesized by seed-mediated growth.[39] Gold seeds and AuNRs were prepared according to the same procedure.[39] Cetrimonium bromide (CTAB) was used as a surfactant in the synthesis of AuNRs. The AuNRs were further purified by centrifugation and redispersed in ethanol. The concentration of AuNRs was determined by UV-visible spectroscopy at 520 nm.

**Results:** The biocompatible AuNR@CaCO$_3$@POPC-AcDX hybrid platform was proved to be biocompatible and had the ability to deliver drugs to HER2 positive cancer cells. The optical density of HER2 (Total) protein was measured at 450 nm using a microplate reader. The optical density of HER2 (pY1248) protein was measured at 450 nm using a microplate reader.

**Figure 8.** Human HER2 (Total) and HER2 (pY1248) detection on SKBR3 cells. a) Human HER2 (Total) detection assay kit on SKBR3 cells by DOX (D), Afatinib (A), 17-AAG (G), DOX+Afatinib (D/A), Afatinib (A), 17-AAG (G), DOX+Afatinib (D/A), 17-AAG+Afatinib (D/A) after 6 h treatment at 37°C. (CaCO$_3$@POPC hybrid particle with 10 $\times$ 10$^{-9}$ M AuNRs was set as control; C$_{DOX}$/C$_{afatinib}$ = 2:1; C$_{DOX}$/C$_{17$-AAG} = 2:1; C$_{G}$/C$_{A}$ = 1:1.) The total drugs concentration is 24 $\times$ 10$^{-9}$ M; the concentration of D/A = G/2 = 12 $\times$ 10$^{-9}$ µM; the concentration of A = 8 $\times$ 10$^{-9}$ µM. The optical density of HER2 (Total) protein was measured at 450 nm using a microplate reader. b) Human HER2 (Total) detection assay kit on SKBR3 cells by DOX (D), anti-HER2 antibody (Ab), 17-AAG (G), DOX+Afatinib (D/A) after 6 h treatment at 37°C. (CaCO$_3$@POPC hybrid particle with 10 $\times$ 10$^{-9}$ M AuNRs were set as control; C$_{DOX}$/C$_{afatinib}$ = 2:1; C$_{DOX}$/C$_{17$-AAG} = 2:1; C$_{G}$/C$_{A}$ = 1:1.) The total drugs concentration is 24 $\times$ 10$^{-9}$ M; the concentration of D/A = G/2 = 12 $\times$ 10$^{-9}$ µM; the concentration of A = 8 $\times$ 10$^{-9}$ µM. The optical density of HER2 (Total) protein was measured at 450 nm using a microplate reader. c) Human HER2 (pY1248) detection assay kit on SKBR3 cells by DOX (D), anti-HER2 antibody (Ab), DOX+Afatinib (D/A) after 6 h treatment at 37°C. (CaCO$_3$@POPC hybrid particle with 10 $\times$ 10$^{-9}$ M AuNRs were set as control; C$_{DOX}$/C$_{afatinib}$ = 2:1; C$_{DOX}$/C$_{17$-AAG} = 2:1; C$_{G}$/C$_{A}$ = 1:1.) The total drugs concentration is 24 $\times$ 10$^{-9}$ M; the concentration of D/A = G/2 = 12 $\times$ 10$^{-9}$ µM; the concentration of A = 8 $\times$ 10$^{-9}$ µM. The optical density of HER2 (pY1248) protein was measured at 450 nm using a microplate reader.
a stabilizer. The morphology of AuNRs was determined by TEM. The AuNRs–CTAB were washed twice by centrifuging at 14 100 rpm for 10 min to remove excess reactants, resuspended in Milli-Q purified water, and kept at room temperature before further experiments. The AuNRs concentration was determined from the UV–vis spectrum ( Cary 100 UV–vis spectrophotometer, Agilent Technologies) and known extinction coefficients. AuNRs concentration in the experiments used was from 1 to 20 × 10−9 M. The ratio between DOX and AuNRs concentration was kept from 2000:1 to 10 000:1.

Incorporation of AuNRs, α-Amylase, FITC-BSA, anti-HER2 Antibody, and Multiple Drugs into the Calcium Carbonate Hybrid Particles: Incorporation of the α-Amylase, anti-HER2 antibody, FITC-BSA, anticancer drug DOX and 17-AAG or DOX and afatinib into the photothermal and pH dual-responsive calcium carbonate hybrid particles was conducted by the following procedure: 1 mL of 5 M CaCl2 and 400 µL of DOX (10 mg DOX/mL H2O) + 500 µL of 5 × 10−9 M AuNRs were gently mixed for 10 min at 800 rpm with magnetic stirring, and then 0.6 mL of 17-AAG or afatinib (6 mg mL−1 in ethanol) and 5 mL of 1 M K2CO3 were added and magnetic stirred at 1600 rpm for 10 min.

After the addition of 5 mL of pure water and discarding the sedimented large particles, the suspension was centrifuged at 15 000 rpm for 15 min, and the supernatant and precipitate were separated. The amount of DOX and AuNRs in the supernatant and precipitate was measured using a UV–vis spectrophotometer (ND-1000 Nanodrop) at 234 nm. Different ratios of CaCO3/K2CO3 were tested as described above.

In Vitro Digestion Study: An in vitro lipolysis model was carried out as described elsewhere.39 Drug-loaded hybrid particles were dispersed in 18 mL of digestion buffer (50 × 10−3 M Trizma maleate, 150 × 10−3 M NaCl, 50 × 10−3 M CaCl2, H2O, pH 7.5) containing 5 × 10−3 M sodium taurocholate hydrate and 1.25 × 10−3 M Egg phosphatidylcholine to simulate the fasted intestinal conditions. The experiment was initiated by adding 2 mL of pancreatin (1000 USP units mL−1) and incubated for 30 min at 37 °C with continuous stirring. 0.2 M NaOH was utilized to keep the pH to a constant value of 7.5 measured with a VWR SB 70P SympHony pH-meter. 180 µL of 0.5 M 4-BPB methanol solution was added to terminate the lipase activity immediately after 1 mL of the reaction mixture was taken and centrifuged at 13 200 rpm for 5 min. The concentration of 17-AAG, Erlotinib, Afatinib, and DOX was determined by LC-MS and UV–vis spectrophotometer, respectively.

Enzyme Activity Assay: The concentration of 17-AAG and afatinib from the release samples at different time intervals and the residue samples was diluted 10–100 times by methanol solution (20% Milli-Q water, 80% methanol, and 0.1% acetic acid) and was analyzed by injecting 20 µL of each diluted sample into an Agilent 1200 LC/MS Sciex 3200 Q-trap tandem MS system in negative electrospray mode for quantification. The transition was monitored at 584/305 nm. The separation was carried out using a 4.6 × 150 mm Supelcosil LC-18 column with 5 µm particle size (Supelco Corp., Bellefonte, PA). The gradient elution was performed with a flow rate of 0.8 mL min−1. The gradient started with 50% B (100% pure methanol) and 50% A (HPLC grade water with 5 × 10−3 M ammonium acetate) for the first 1 min, ramping to 80% B in 0.01 min, and then linearly increasing to 100% B over 4.5 min. The 100% B mobile phase was then held for 1 min before decreasing to 50% B over 2.5 min. This resulted in an 8 min total run time, including reconditioning of the column. The 17-AAG concentration and afatinib in each sample were calculated with the calibration curves y = 11.25x + 44.463 (R2 = 0.9832) and y = 13.478x – 46.523 (R2 = 0.9922), respectively, which was developed by injecting standard solutions into the LC-MS with known amount of 17-AAG and afatinib standards (25, 50, 125, 250, and 500 ng mL−1). The standard solution contained the same percentage of release medium and methanol solution as of each sample.

In Vitro Cytotoxicity Studies: HeLa (a human cervical carcinoma cell line), MCF-7 (a human breast cancer cell line), MDA-MB-231 (human adenocarcinoma cell line), SKBR-3 (HER2 positive breast cancer cells), and M28 (a human mesothelioma cell line) were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with
10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin. The cell medium was filtered through 0.2 µm filters. 100 µL cell medium containing HeLa cells, MCF-7 cells, and the human epidermal growth factor receptor 2 (HER2) overexpressed cell line SKBR-3 with or without calcium carbonate hybrid particles in PBS at different concentrations (10, 50, and 100 µg mL$^{-1}$) was seeded on black 96-well plates (1.0 × 10⁴ cells per well) and incubated 24 h with and without 10% (v/v) AlamarBlue in an atmosphere of 5% CO₂ at 37 °C. The fluorescence intensity was tested at the excitation and emission wavelengths of 560 and 590 nm, respectively, for the AlamarBlue assay in a fluorescence microplate reader by SpectraMax M2 or i3 (Molecular devices, USA). The cell viability of blank calcium carbonate, blank AuNRs@CaCO₃@POPC, AuNRs@CaCO₃@POPC@AcDX, and therapeutics loaded CaCO₃@ POPC and CaCO₃@POP@AcDX and on HeLa, M28, MCF7, SKBR3, and MCF-7/DX cells was calculated based on the fluorescence intensity according to following equation: cell viability = ($F_1 - F_0$)/($F_0 - F_b$). 100 µL of cell medium containing MCF-7 cells with or without single/multiple drug-loaded calcium carbonate suspensions at different concentrations (100, 200, 500, and 1000 ng mL$^{-1}$) was seeded on black 96-well plates (1.0 × 10⁴ cells per well) and incubated for 24 h with and without 10% (v/v) AlamarBlue in an atmosphere of 5% CO₂ at 37 °C. The cell viability of single/multiple drug-loaded calcium carbonate suspensions on MCF-7 cell lines was measured and calculated based on three independent experiments of each negative control, blank control, and samples in the same way as the blank calcium carbonate particles without drugs. The same protocol was used according to our previous work. [33,34]

Live and dead assay: 100 µL of 1 × 10⁴ µ calcein AM × 2 × 10⁻⁶ µ of ethidium homodimer-1 (EthD-1) culture medium was added to 100 µL cell medium containing either HeLa, MCF-7 cells, MCF-7/DXO, or SKBR-3 cells with or without particles seeded on 96-well culture plates at a density of 1 × 10⁴ cell mL$^{-1}$ in an atmosphere of 5% CO₂ at 37 °C and cultured for 24 h. The cell number and density was counted using an Invitrogen Countess automated cell counter. The fluorescence intensity was measured at the excitation wavelengths of 488 and 544 nm and the emission wavelengths of 530 and 610 nm for calcein AM and EthD-1, respectively, using a microplate reader SpectraMax i3 (Molecular device). The cell viability of hybrid particles on HeLa, MCF-7, MCF-7/DXO, or SKBR-3 cell lines was calculated according to assay described in our previous work. [33]

Multidrug Resistance Test: 96-well plates (Costar, Corning Corp.) with clear bottom were used to study the multidrug resistance of single, multiple drugs therapeutics combination incorporated into calcium carbonate hybrid particles coated with and without pH sensitive polymer AcDX and on HeLa, M28, MCF7, SKBR3, and MCF-7/DXO cells. The cytotoxicity of HeLa cells after 4 h treatment according to standard assay protocol. HeLa cells with density of 10⁴ cells per well were seeded on 12-well cell culture plate and treated with different therapeutics for 16 h. The optical density at 450 nm of the standards and samples according to the assay kit standard protocol was obtained using a microplate reader (SpectraMax i3 from Molecular device).

Human HER2 (pY1248) ELISA assay was used to detect and quantify the phosphorylated HER2 (pY1248) protein of its phosphorylation state from SKBR3 lysates treated with different therapeutics with and without anti-HER2 antibody. SKBR3 cells with density of 10⁵ mL$^{-1}$ were seeded on 12-well cell culture plate and treated with different therapeutics for 12 h. The optical density at 450 nm of the standards and samples according to the assay kit standard protocol was obtained using a microplate reader (SpectraMax i3 from Molecular device).

Human Plasma Stability: The particles were suspended in PBS, pH 7.4 at concentration of 1 mg mL$^{-1}$. Then 100 µL of the solution was added to 900 µL of human plasma. The samples were incubated at 37 °C with magnetic stirring. At 1, 2, 5, 10, 15, 20, 40, 60, 90, and 120 min, 50 µL of each sample was taken and diluted to 950 µL of mQ H₂O. The particle size, PDI, and Zeta potential were measured by Malvern Zeta Sizer.

Incubations of Aftatinib incorporated calcium carbonate coated with and without polymer AcDX were carried out in 96-well cell culture plate (Corning) in five aliquots of 100 µL each (one for each time point). Test compounds (10 × 10⁻⁶ µ, final solvent concentration 1%) and DMSO (10 × 10⁻³ µ, 0.05% DMSO) were incubated at 37 °C for five time points over 120 min were analyzed (0, 15, 30, 60, and 120 min). All incubations were performed in duplicates. The samples were analyzed by HPLC-MS (API3000, AB Sciex). The percentage of parent compound remaining after incubation in human plasma was plotted versus incubation time.

Statistical Analyses: The data were analyzed by a Student’s t-test using the online version of GraphPrism software. The significance was set at probabilities of *p < 0.05, **p < 0.01, and ***p < 0.001.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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